Novel role of aaRS in tRNA biology in *S. cerevisiae*

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Abstract

Aminoacyl tRNA synthetases (aaRS) are enzymes that add particular amino acids to cognate tRNAs. These aaRS are essential for the housekeeping function of protein synthesis. We recently reported that temperature sensitive (ts) mutantions of some of the genes encoding aaRSs are defective in pre-tRNA splicing and accumulate end-processed intron containing $tRNA^{Ile}_{UAU}$ in *S. cerevisiae* at the non-permissive temperature. These previous studies identified 5 mutant aaRS genes: *ils1, gln4, ths1, frs2, and cdc60* (Wu et al, 2015). My further analysis showed *ala1* also causes accumulation of unspliced $tRNA^{Ile}_{UAU}$, but eleven other ts aaRSs do not. The splicing defect is surprising since aaRSs are not known to affect tRNA processing steps. This gives rise to the idea that aaRSs may possess novel activities in tRNA biosynthesis. I am conducting a systematic analysis utilizing a non-radioactive northern method for all aaRS mutants which have been isolated, 16 out of the 20 aaRS, to determine which aaRS may affect any of the 10 families of tRNAs that are encoded by intron-containing genes. In addition, I am testing three possible mechanisms by which faulty aaRS would cause defects in pre-tRNA intron removal: defective tRNA nuclear export to the cytoplasm, failure to deliver tRNA to mitochondria where intron splicing takes place in yeast, or defects in the splicing mechanism itself. Studies employing fluorescent *in situ* hybridization (FISH) techniques investigated the possibilities by determining the subcellular location of the unspliced tRNAs. They were not located in the nucleus, which leads to the conclusion the defect is most likely not due to inefficient tRNA nuclear export. This leaves the other alternative two

possibilities mentioned above. Completion of the study will offer insight to the tRNA biological pathway and a novel role of the aaRS.

Introduction

Traditionally tRNAs have been considered to be passive decoders of genetic information with their main role being involved in protein synthesis. Recent research has shown tRNAs are also involved in other major cellular processes, such as cellular response to stress, apoptosis, protein degradation, retrovirus replication, and cancer formation. (Phizicky and Hopper, 2010) Despite decades of research, there is still a lack of knowledge about tRNA biogenesis and subcellular dynamics.

Transcription of tRNA occurs inside of the nucleus generating initial transcripts (P) that consist of both 5' and 3' leading and trailing sequences. Ten out of the 42 yeast tRNA families also encode an intron (Hani and Feldmann 1997). The leading and trailing sequences are removed within the nucleus and certain modifications as well as the CCA 3' nucleotides are added to the tRNA. These end-processed, partially modified tRNAs are then exported into the cytoplasm. Nuclear export is mediated via the exporter, Los1 (exportin-t) and other uncharacterized exporters. The intron-containing tRNAs (I) are then reach the mitochondria outer surface where the introns are removed (Yoshihisa et al 2003). Additional modifications are added in the cytoplasm to produce the mature tRNA (M) which can then be charged with an amino acid and function in translation (Phizicky and Hopper, 2010).

In an attempt to identify all missing gene products that are involved in tRNA biology, our laboratory conducted an unbiased genome-wide screen of nearly all annotated protein-encoding genes in *S. cerevisiae,* (budding yeast) (Wu et al 2015). The screen identified temperature sensitive (ts) mutations of genes encoding five of the tRNA aminoacyl synthetases (aaRS). Each of these five mutants—*ils1, gln4, ths1, frs2, and cdc60* - accumulated intron-containing $tRNA^{Ile}_{UAU}$. This accumulation indicates that there is defect in the ability of the intron to be spliced out (Wu et al 2015). As the primary role of the aaRS occurs after the intron has been removed, it is likely that the aaRS possess a novel role in earlier tRNA processing steps that results in a splicing defect when the genes are mutant.

tRNA charging with an amino acid is catalyzed by the aaRS. aaRS are divided into two classes. Class I typically consist of a monomer that possesses a Rossman fold. Class II possesses a unique fold found only in the class II aaRS, a biotin synthetase holoenzyme. Most of the class II enzyme form homodimers from two sister structures, as is the case with Frs1 and Frs2 for PheRS (Ibba and Söll, 2000).

There is one synthetase per amino acid. Each identified the appropriate tRNA by particular nucleotides, often located at the anticodon. Aminoacylation is a two step reaction. ATP is required for step one. Both the amino acid and an ATP bind at the active site of the aaRS. A nucleophilic displacement mechanism occurs between the α-phosphate on the ATP and the α-carboxylate on the amino acid. This forms an aminoacyl-adenylate. In step two the aaRS then will bind the

cognate tRNA and the aminoacyl-adenylate can nucleophilically attack the hydroxyl on the terminal adenine of the CCA group. This will result in the esterfication of the tRNA with the aminoacid, creating the bond, and an AMP to be released. The tRNA amino acid ester linkage is often unstable at neutral pH and tRNAs must be kept at a pH less than or equal to 4 in order to stabilize the bond (Ibba and Söll, 2000).

Recent studies have documented novel functions for aaRS. Examples of these functions include: regulation of glucose and amino acid metabolism, tissue and organ development, angiogenesis for vasculature, inflammatory responses, cell death, cell stress, and immune response (Guo and Schimmel 2013). Most of the novel functions are encoded in extra domains in the genes encoding aaRS in human cells that are not in the prokaryotic homologs. Nine of the 20 *S. cerevisiae* aaRS genes also encode extra domains (Guo et al 2010). The functions of these domains in the yeast protein have yet to be explored.

The goal of this research project was to discover the mechanism by which mutations in aaRS could affect pre-tRNA splicing. To reach this goal the available aaRS ts mutants were investigated for their affect on the tRNA processing pathway for all intron-containing tRNA. I learned that a subclass of the aaRS cause accumulation of several non-cognate end processed intron-containing tRNAs in the cell. In order to narrow down the possible mechanisms of mutant aaRS that lead to defective tRNA splicing fluorescent *in situ* hybridization was utilized to localize tRNA. The data supports a cytoplasmic mechanism and not a nuclear export defect.

Materials and Methods

Strains and Media

Temperature sensitive mutants for 16 aaRS genes are available in the ordered collections from Dr. C. Boone (ArgRS, AsnRS, CysRS, GluRS, GlnRS, GlyRS, PheRS, ProRS, ThrRS, and ValRS) and Dr. P. Hieter (AlaRS, HisRS, IleRS, LeuRS, MetRS, and TyrRS) (Ben-Aroya S, *et al.*, 2008 and Li Z, *et al.*, 2011). YEPD media (yeast extract, trypticase peptone, dextrose, supplemented with adenine [0.04g/L], uracil [0.04g/L]) was used for all experiments.

Small RNA isolation

Yeast strains were grown in 96 deep-well plates to early log phase and then grown in 50mL flasks at permissive temperature of 23°C until an OD_{600} of 0.2-0.4 was reached, then the cells were shifted to 37°C for two hours before the RNA extraction. Small RNAs were isolated using the method described in Wu et al (2013).

Northern Analysis

tRNAs were analyzed by use of a non-radioactive Northern method utilizing DIG-labeled probes (Table 1) as described in Wu et al (2013). 2µL of RNA was added per lane and the gel was run from 105-110V for 21-22 hours. The wild type parental strain was BY4741 and *sen2-42* (Ben-Aroya S, *et al.*, 2008) which carries a ts mutation in the gene encoding the Sen2 subunit of the tRNA splicing endonuclease (Yoshihisa et al 2007), was employed as a positive control. Data

acquired using a Lumi-imager in combination with a LumiAnalyst program. Quantitation completed using Image J program.

Fluorescent *in situ* hybridization

Cells were grown in 15 mL of liquid media overnight at 23 °C to early log phase (OD_{600} = 0.15-0.3). Temperature-sensitive strains were shifted to 37 °C for 2 hours before OD_{600} reached 0.3. Cells were collected and fluorescence *in situ* hybridization (FISH) was performed as previously described using probe SRIM03 and SRIM04 for $tRNA^{Ile}_{UAU}$ (Sarkar and Hopper, 1998) Images were captured by using a Nikon 90i with a CoolSNAP HQ2 digital camera and analyzed using NIS-Elements (Nikon, Melville, NY). Images were assembled using the Adobe Photoshop C5.

Results

Analysis of the affect of aaRS ts mutantions in splicing of intron-containing tRNAs

Previous studies discovered intron-splicing defects for several aaRS ts mutants by analyses of the affects on mutant genes upon $tRNA^{Ile}_{UAU}$ (Wu et al 2015). $tRNA^{Ile}_{UAU}$ was chosen for the genome-wide screen because the gene encoding $tRNA^{Ile}_{UAU}$ contains the largest intron (60nt), facilitated analysis of all of the tRNA processing intermediates. tRNA intermediates that were scored were: the initial pre-tRNA transcript (P) that contains 5' leader, 3' trailer, and intron; the end-processed unspliced form (I); mature tRNA (M); (Figure 1) free introns; and free exons. In order to determine whether the other 9 families of intron-containing

tRNAs were affected similarly by the various lesions of aaRS a non-radioactive northern analysis using DIG labeled probes was employed (Wu et al 2103). As the laboratory has only conducted similar research using $tRNA^{Ile}_{UAU}$, I designed DIG-labeled probes complementary with both the exon and intron for each tRNA in order to detect the P, I, and M intermediate stages of tRNA processing. The sequences of the oligos are provided in Table 1 and the regions of the tRNAs complementary to the probes are depicted in Figure 2. Each of the 9 tRNA families with intron-containing genes was examined 3 times using northern analysis, with each examination utilizing newly isolated RNA extracted from independent cultures. If a defect in ptr-tRNA splicing occurs, the end-processed, intron-containing species will accumulate and the ration of I/P will be greater for the mutant than the tRNA isolated from wild-type parental cells (Figure 1, lane 2).

tRNAs from the 10 intron encoding tRNA families were assessed in 16 aaRS ts mutants strains that were available from the ts mutant collections created by Boone and Hieter groups. Mutations of the remaining 4 genes encoding aaRS are unavaialable in any mutant collection. Analysis of aaRS was undertaken for three reasons: (1) to determine if the ts aaRS caused a splicing defect for cognate tRNA, (2) to test whether defective aaRS caused splicing defects in noncognate tRNAs, and (3) to determine which of the aaRS affect pre-tRNA splicing.

Analysis of $tRNA^{Ile}_{UAU}$

I first confirmed that the mutants reported previously (Wu et al 2015) consistently showed accumulation of end-processed intron-containing $tRNA^{Ile}_{UAU}$

(Figure 3). As anticipated the five original aaRS mutants –*ils1* (IleRS)*, ths1* (ThrRS)*, frs2* (PheRS)*, gln4* (GlnRS)*, and cdc60* (LeuRS) showed accumulation of the end-processed intron-containing tRNA. Other aaRS mutants provided variable results such as *ala1*. Further studies are required to assess whether additional aaRS cause a splicing defect in $tRNA^{Ile}_{UAU}$.

Analysis of $tRNA^{Tyr}_{GUA}$

$tRNA^{Tyr}_{GUA}$ has the smallest intron (14nt) of the tRNA intron-containing gene families. The short sequence made it difficult to design a probe that could specifically hybridize to the pre-$tRNA^{Tyr}_{GUA}$ as analyzed by the Northern method. Therefore, an alternative probe was designed that is complementary to regions including an extended 5'exon sequence as well as part of the 3'exon was designed to conduct Northern analyses for $tRNA^{Tyr}_{GUA}$ (Figure 2). However, even employing the re-designed probe, it was difficult to consistently assess the affects of the aaRS mutations on splicing of pre-$tRNA^{Tyr}_{GUA}$ (Figure 4). Although in additional studies it appears that *YDR341C* (ArgRS)*, ded81* (AsnRS)*, YNL247C* (CysRS)*, grs1* (GlyRS)*, hts1* (HisRS)*, ils1, frs2,* and *ths1* as aaRS mutants cause moderate defects in pre-tRNA splicing (Table 3), further studies will be necessary.

Analysis of $tRNA^{Phe}_{GAA}$

Northern analyses of $tRNA^{Phe}_{GAA}$ (Figure 4) generated unanticipated extra RNA species. The extra species rendered analyses somewhat difficult. Although the identities of the extra RNA species are unknown, they could be precursors with slowed 5' or 3' processing. In spite of this difficulty, it was

possible to identify mutants that consistently accumulated 5' and 3' processed intron-containing pre-tRNA: *gln4, grs1, frs2,* and *ths1* (Table 3). Other aminoacyl tRNA synthetase mutant's *mes1* (MetRS) and *vas1* (ValRS) showed variable results. Further studies employing alternative probes are required in order to assess the role of the aaRS in splicing of $tRNA^{Phe}_{GAA}$.

Analysis of $tRNA^{Trp}_{CCA}$

The $tRNA^{Trp}_{CCA}$ probe consistently hybridizes to a tRNA species migrating slower than the end-processed intron-containing tRNA (Figure 6). According to an unpublished report (A.K. Hopper, personal communication), tryptophan tRNA is encoded by two genes that initiate transcription at sites giving rise to different lengths of the 5' leaders. To determine the levels of initial pre-tRNA transcripts (P) compared to end-processed intron-containing intermediates (I) both the slow and faster migrating bands were included. The results indicate that *gln4, frs2, ths1, ils1,* and *cdc60* mutants affect pre-$tRNA^{Trp}_{CCA}$ splicing (Table 3). The cause of the accumulation of the precursor band in select aaRS mutants is unknown and requires further study.

Analysis of $tRNA^{Leu}_{CAA}$

Similar to $tRNA^{Phe}_{GAA}$, Northern analysis of $tRNA^{Leu}_{CAA}$ contained unanticipated signals migrating slower than end-processed intron-containing tRNA (Figure 7). The undefined RNA species may be caused by slowed 5' or 3' processing which generally occurs simultaneously. Analysis of the mutant aaRS determined that *YDR341C, gln4, hts1, ils1, cdc60, frs2,* and *ths1* have defects in pre-$tRNA^{Leu}_{CAA}$ splicing (Table 3). In addition *ala1, ded81, YNL247W,* and

YHR020W showed defects in splicing in some, but not all, of the studies (Figure 7). Additional studies are required to evaluate the roles of AlaRS, AsnRS, CysRS, and ProRS on splicing of pre-tRNA$^{Leu}_{CAA}$.

Analysis of tRNA$^{Leu}_{UAG}$

Analysis of tRNA$^{Leu}_{UAG}$ was straightforward as the Northern pattern of tRNAs was as anticipated. The repeat analysis showed that *gln4, hts1, ils1, frs2, and ths1* consistently accumulated end-processed intron-containing pre-tRNA$^{Leu}_{UAG}$ (Figure 8). In analyses of tRNA$^{Leu}_{UAG}$ from *mes1* cells yielded variable results and will need to be investigated further.

Analysis of tRNA$^{Pro}_{UGG}$

Similar to tRNA$^{Trp}_{CCA,}$ tRNA$^{Pro}_{UGG}$ is encoded by two genes that generate initial transcripts with 5' leaders of different lengths, thus leading to a slower migrating band (O'Conner and Peebles, 1991). To determine the levels of initial pre-tRNA transcripts (P) compared to end-processed intron-containing intermediates (I) both the slow and faster migrating bands were included. Analysis of tRNA$^{Pro}_{UGG}$ demonstrated that *gln4, frs2,* and *ths1* consistently caused accumulation of end-processed intron-containing tRNA. In contrast, analysis of *mes1, ils1*, and *cdc 60* produced inconsistent results and will need to be studied further (Figure 9 A-C).

An unanticipated RNA species migrating faster than the mature tRNA$^{Pre}_{UGG}$, labeled as '?', was detected by Northern analyses using the tRNA$^{Pro}_{UGG}$ probe. To determine the identity of the unanticipated fast migrating RNA species, I designed three additional probes. I generated probes

complementary to just the 5'exon, the intron, and the 3'exon. Analysis employing the intron probe demonstrated that the unknown RNA species does not contain the intron sequence (Figure 9 D). Analysis employing the 5' exon demonstrates that the sequence hybridizes to both the anticipated slow species and the faster migrating species (Figure 9 E). In contrast the 3'exon probe hybridizes strongly to the mature tRNA slower species and poorly to the faster migrating unknown RNA (Figure 9 F). The extra RNA species is also present in cells not exposed to heat shock (Figure 9 E and F, lane 6). Further analysis will be needed in order to identify this unanticipated fast migrating $tRNA^{Pro}_{UGG}$ species.

Analysis of $tRNA^{Lys}_{UUU}$

Similar to both $tRNA^{Trp}_{CCA}$ and $tRNA^{Pro}_{UGG}$, $tRNA^{Lys}_{UUU}$ is encoded by genes that generate initial transcripts with 5' leaders of different lengths (O'Conner and Peebles, 1991). To determine the levels of initial pre-tRNA transcripts (P) compared to end-processed intron-containing intermediates (I) both the slow and faster migrating bands were included. Three mutants: *gln4, frs2,* and *ths1* consistently show defects in splicing of end-processed intron-containing pre-$tRNA^{Lys}_{UUU}$ compared to the WT and other aaRS (Figure 10).

Analysis of $tRNA^{Ser}_{CGA}$

The initial probe designed to hybridize to the end-processed intron-containing $tRNA^{Ser}_{CGA}$ did not detect the mature tRNA species. Therefore, I generated an additional probe that contained the entire 5'exon. Analysis employing the new probe showed, with the exception of *frs2,* none of the aaRS mutants appeared to affect splicing of $tRNA^{Ser}_{CGA}$ (Figure 11).

Analysis of $tRNA^{Ser}_{GCU}$

Similar to $tRNA^{Ser}_{CGA}$ initial probe designed to hybridize to the end-processed intron-containing $tRNA^{Ser}_{CGA}$ did not detect the mature tRNA species and again a new probe that extended to hybridize with the entire 5'exon was generated. Analysis employing the new probe showed that none of the aaRS mutants appear to cause a splicing defect in $tRNA^{Ser}_{GCU}$ (Figure 12).

Overall, after examination of all of the intron-containing tRNA families, it can be concluded that a subset of the mutant aaRS - *gln4, frs2, ths1* – appear to affect pre-tRNA splicing for most of the tRNA families encoded by intron-containing genes.

Examination of tRNA nuclear export

A goal of this study was to determine which step in tRNA biology caused this defective splicing to take place. There are at least three possible steps: (1) nuclear export of the end-processed intron-containing tRNA; (2) transport of the end-processed intron-containing tRNA to the mitochondrial outer surface, and (3) removal of the intron catalyzed by the tRNA splicing complex localized on the outer surface of the tRNA. Localizing pre-tRNAs to either inside the nucleus or in the cytoplasm of cells with mutant aaRS will help narrow down the possible tRNA biology steps.

Fluorescent *in situ* hybridization (FISH) was employed to determine where the tRNAs are located within the cells in the mutants as compared to the wild-type cells. If the mutant aaRS causes a defect in nuclear export, the FISH will generate tRNA nuclear accumulation. Nuclear accumulation is viewed in the

positive control *rna1-1* as it encodes a mutant in the RanGAP pathway that disrupts nuclear-cytoplasmic exchange and causes accumulation of unspliced tRNA in the nucleus (Sarkar and Hopper, 1998).

If the tRNAs are instead localized primarily in the cytoplasm, the mutant aaRS either affect the tRNA transport to the outer surface mitochondria or the ability of the intron to be removed from tRNA via defective splicing on the surface of mitochondria. Cytoplasmic tRNA is viewed in *sen2-42,* which has a pre-tRNA splicing defect as a result of a mutation in the Sen2 subunit of the splicing endonuclease (Yoshihisa et al 2007).

In collaboration with Dr. Shubhra Majumber, the location of $tRNA^{Ile}_{UAU}$ was studied as reliable probes (Table1) were already created. SRIM03 hybridizes to mature $tRNA^{Ile}_{UAU}$ and allows localization of all the different tRNA species that were examined by Northern analysis. SRIM04 hybridizes to only $tRNA^{Ile}_{UAU}$ intron, which allows examination of the localization of the initial-precursor tRNA (P) and the end-processed intron-containing tRNA (I) species , but not the mature band.

In addition, the pre-$tRNA^{Ile}_{UAU}$ splicing defect that occurs with mutations in a subset of the aaRS is consistent. The mutant PheRS, *frs2*, was chosen as the mutant strain as the mutant generates one of the strongest accumulations of end-processed intron-containing $tRNA^{Ile}_{UAU}$.

FISH analysis detected no nuclear accumulation for the *frs2* mutant strain for either the mature sequence and intron sequence (Figure 13 A and B row 4). This suggests that the mutant aaRS does not affect $tRNA^{Ile}_{UAU}$ nuclear export

and instead the $tRNA^{Ile}_{UAU}$ splicing defect may be caused by an inability of tRNA to be transported to the mitochondria outser surface or the efficiency of the splicing by the endonuclease on the mitochondria outer surface. In addition, the tRNAs appears to form clumps in the cytoplasm (Figure arrows). What these clumps are is completely unknown. Northern analyses are required to verify that accumulation of unspliced pre-$tRNA^{Ile}_{UAU}$ utilizing the same culture of cells employed for the FISH analysis. Studies need to be extended to other aaRS mutants and other tRNAs. If the lack of nuclear accumulation of end-processed intron-containing tRNAs is consistent, one would conclude that defect in pre-tRNA splicing caused by aaRS mutations is due to a post nuclear export step.

Discussion

aaRS that accumulate intron-containing tRNA

A goal of this study was to determine which mutant aaRS cause a defect in splicing of end-processed intron-containing tRNAs in yeast. Three mutants appear to have a general role in the splicing of most pre-tRNAs. These are *gln4, ths1, and frs2.* Why mutations of these particular three aaRS result in defective pre-tRNA splicing is unclear, but here I consider 2 possibilities. There are 2 classes of aaRS enzymes, I and II, each of which contain 10 members. The two different aaRS classes have different folding structures and different ways of binding the cognate aaRS. Moreover, the aaRS mutants that cause a pre-tRNA splicing defect do not belong to the same class: Gln4 is class I while Ths1 and Frs2 are class II (Ibba and Söll 2000). This eliminates the possibility of the novel function is unique to one specific class of aaRS. However, many eukaryotic

aaRS, regardless of class, contain extra domains with novel functions not found in the prokaryotic counterparts. Interestingly, all three of the aaRSs which were shown to play a role in pre-tRNA splicing contain an additional unique domain found in only 5 of the yeast aaRS: CysRS, GlnRS, ThrRS, PheRS, and AsnRS (Guo et al 2010). It remains to be learned what these unique domains encode and if they may encode activities that regulate pre-tRNA processing. Future studies to map the ts region within the relevant mutant aaRS may prove to be informative. If the region maps to the extra domain contains the mutation perhaps swap-type studies could address the function. If the mutation maps elsewhere, the importance of that region would need to be determined to try and understand how it is causing the accumulation of unspliced pre-tRNAs.

Mechanism by which aaRS may affect pre-tRNA splicing

Our data support the model that a subset of aaRSs have a function in a pre-tRNA splicing in addition to the aminoacylation of tRNAs. Defective aminoacyl tRNA synthetases that cause defects in splicing of non-cognate tRNAs needs to be studied further. One potential explanation of the non-cognate affect of mutant aaRS on tRNAs is that the mutant aaRS lead to a defect in aminoacylation of multiple tRNAs. To study this an acid-urea gel was conducted by Dr. Kunal Chatterjee for $tRNA^{Ile}_{UAU}$ for mutant aaRS *ths1* (Figure 14, Unpublished). tRNA aminoacylation is unstable at a pH of less than or equal to 4. The gel includes tRNA in a pH of 4.4 and a pH of 7.5. When most of the tRNA is properly aminoacylated, in pH of 4.4 only one tRNA species is produced. When more tRNAs are not aminoacylated, like they are in non-acidic conditions, pH of

7.5, two species of tRNA are viewed. If the mutant aaRS *ths1* produces a single tRNA species at pH of 4.4 at non-permissive temperature, then there is no defect in aminoacylation for $tRNA^{Ile}_{UAU}$. If there are two species of tRNA, then the mutant aaRS causes an aminoacylation of the non-cognate tRNA. The acid-urea gel generated a single $tRNA^{Ile}_{UAU}$ species for the ThrRS mutant when at the non-permissive temperature and in the favorable pH condition. These results support that *ths1* does not affect the ability of the isoleucine to attach to $tRNA^{Ile}$. More acid-urea gels were conducted for aaRS mutants *frs2, cdc60,* and *gln4* (data not shown). These also produced similar results to that of *ths1*.

While the acid-urea gel analysis may eliminate the possibility of a charging defect causing a feedback loop causing a pause in tRNA processing, it does not eliminate the possibility of a negative feed back loop. The inability of the introns to be spliced out because of the aaRS may lead to a block in the transcription of new pre-tRNAs. This could explain why in many of the gels the initial precursor tRNA appeared to be in low quantities.

The reasoning behind the mechanism for the pre-tRNA splicing defect is still unknown but the FISH analysis for *frs2* did show that there is no detectable nuclear accumulation of either mature or intron-containing $tRNA^{Ile}_{UAU}$ which argues against the possibility of a defect in pre-tRNA nuclear export. The lack of nuclear accumulation also provides support the concept that the novel function of tRNA aaRS is a cytoplasmic role. This data still needs to be confirmed by northern analysis to ensure the accumulation of unspliced pre-tRNA is still present. FISH also needs to be conducted for the other ts aaRS mutants that

cause the accumulation defect as well as for the ts aaRS mutants that do not cause the defect to prove that this is consistent.

In addition, it needs to be determined if the clumps that appear in the cytoplasm for the *frs2* FISH data also occurs in other mutants. What the clumps of tRNA exactly are and if they are occurring in specific areas are still unknown. A possibility is that the clumps consist of both the tRNAs and the tRNA aminoacyl synthetases. If both are present it could mean that the tRNA aminoacyl synthetase is somehow binding the tRNAs and pulling them into a clump, preventing their ability to reach the mitochondria and being properly spliced.

The analysis of the aaRS ts mutants showed inconsistencies that need to be addressed for the future. The varying appearance of unspliced pre-tRNA accumulation should be addressed by more repeats in order to determine if the accumulation consistently occurs. The unspliced pre-tRNA accumulation could have occurred with a mistake in the RNA isolation process as many yeast strains were being isolated at once. A final issue is concerning the quantitative analysis. It appeared to have inconsistencies and inaccuracies. The qualitative issues are caused by the quality of the northern under analysis. Many of the northern analyses have lines or streaks that are causing problems with determining accurate values. *sen2* has a strong unspliced pre-tRNA accumulation creating a strong signal that bled into the adjacent lanes. In retrospect it should not be loaded into immediately adjacent lanes. I amend this in the future. More repeats of the Northerns taking this into consideration and possible a new method of quantitative could address these issues.

My study showed a subset of mutants aaRS which cause a defect in intron splicing of pre-tRNAs that may be caused by a aaRS novel function. In addition, the studies argue against the possibility of the aaRS playing a role in initial pre-tRNA nuclear export.

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Figure 1 Cartoon of Northern analysis depicting accumulation of end-processed intron-containing tRNA (Lane 2) as compared to WT function (Lane 1) Modified from Wu et al 2015

Figure 2. tRNA DIG probe complementary sequences with predicted secondary structures of pre-tRNA intron-containing families images modified from Ogden, Lee and Knapp 1984. From left to right. Top row: $tRNA^{Ile}_{UAU}$, $tRNA^{Tyr}_{GUA}$ $tRNA^{Phe}_{GAA}$. Middle row: $tRNA^{Trp}_{CCA}$, $tRNA^{Leu}_{CAA}$, $tRNA^{Leu}_{UAG.}$ Bottom row: $tRNA^{Pro}_{UGG}$, $tRNA^{Lys}_{UUU}$, $tRNA^{Ser}_{CGA.}$ $tRNA^{Ser}_{GCU}$ not depicted: Complementary to complete 5'exon and full intron. Introns contain sequence marked by black arrows.

Figure 3 Mutations in GlnRS, IleRS, LeuRS, PheRS and ThrRS cause accumulation of end-processed intron-containing $tRNA^{Ile}_{UAU}$. Northern analysis of $tRNA^{Ile}_{UAU}$ employing DIG probe JW0048. Initial precursor transcript tRNA (P), end-processed intron-containing tRNA (I), Mature tRNA (M). I/P ratio of species I divided by P and normalized to wild-type cells grown under same conditions. Lane numbers marked in red are mutant aaRS which consistently cause accumulation of end-processed intron-containing tRNAs.

Figure 4 Mutations in ArgRS, AsnRS, CysRS, GlyRS, HisRS, IleRS, PheRS and ThrRS cause accumulation of end-processed intron-containing $tRNA^{Tyr}_{GUA}$. Northern analysis of $tRNA^{Tyr}_{GUA}$ employing DIG probe KC0031. Initial precursor transcript tRNA (P), end-processed intron-containing tRNA (I), Mature tRNA (M). I/P ratio of species I divided by P and normalized to wild-type cells grown under same conditions. B is not normalized to wild-type but ratios are compared to

each other to draw conclusions. Lane numbers marked in red are mutant aaRS which consistently cause accumulation of end-processed intron-containing tRNAs.

Figure 5 Mutations in GlnRS, GlyRS IleRS, LeuRS, PheRS and ThrRS cause accumulation of end-processed intron-containing $tRNA^{Phe}_{GAA}$. Northern analysis of $tRNA^{Phe}_{GAA}$ employing DIG probe SM10. Initial precursor transcript tRNA (P), end-processed intron-containing tRNA (I), Mature tRNA (M). I/P ratio of species I divided by P and normalized to wild-type cells grown under same conditions. B) lanes 1-4 could not acquire I/P ratios accurately so conclusions drawn from A and C. Lane numbers marked in red are mutant aaRS which consistently cause accumulation of end-processed intron-containing tRNAs.

Figure 6 Mutations in GlnRS, IleRS, LeuRS, PheRS and ThrRS cause accumulation of end-processed intron-containing $tRNA^{Trp}_{CCA}$. Northern analysis of $tRNA^{Trp}_{CCA}$ employing DIG probe SM11. Slower migrating initial precursor transcript tRNA (Pa), Faster migrating initial precursor transcript (Pb), end-processed intron-containing tRNA (I), Mature tRNA (M). I/P ratio of species I divided by (Pa+Pb) and normalized to wild-type cells grown under same conditions. Lane numbers marked in red are mutant aaRS which consistently cause accumulation of end-processed intron-containing tRNAs.

Figure 7 Mutations in ArgRS, GlnRS, HisRS, IleRS, LeuRS, PheRS and ThrRS cause accumulation of end-processed intron-containing $tRNA^{Leu}_{CAA}$. Northern analysis of $tRNA^{Leu}_{CAA}$ employing DIG probe SM12. Initial precursor transcript tRNA (P), end-processed intron-containing tRNA (I), Mature tRNA (M). I/P ratio of

species I divided by P and normalized to wild-type cells grown under same conditions. B is not normalized to wild-type but ratios are compared to each other to draw conclusions. Lane numbers marked in red are mutant aaRS which consistently cause accumulation of end-processed intron-containing tRNAs.

Figure 8 Mutations in GlnRS, HisRS, IleRS, PheRS and ThrRS cause accumulation of end-processed intron-containing $tRNA^{Leu}_{UAG}$. Northern analysis of $tRNA^{Leu}_{UAG}$ employing DIG probe SM13. Initial precursor transcript tRNA (P), end-processed intron-containing tRNA (I), Mature tRNA (M). I/P ratio of species I divided by P and normalized to wild-type cells grown under same conditions. B is not normalized to wild-type but ratios are compared to each other to draw conclusions. Lane numbers marked in red are mutant aaRS which consistently cause accumulation of end-processed intron-containing tRNAs.

Figure 9 A-C) Mutations in GlnRS, PheRS and ThrRS cause accumulation of end-processed intron-containing $tRNA^{Pro}_{UGG}$. Northern analysis of $tRNA^{Pro}_{UGG}$ employing DIG probe SM14. Slower migrating initial precursor transcript tRNA (Pa), faster migrating initisl precursor transcript, end-processed intron-containing tRNA (I), Mature tRNA (M), Unknown fast migrating RNA species (?). I/P ratio of species I divided by (Pa+Pb) and normalized to wild-type cells grown under same conditions. A is not normalized to wild-type but ratios are compared to each other to draw conclusions. B is missing *ala1-1* so conclusions drawn from A and C. Lane numbers marked in red are mutant aaRS which consistently cause accumulation of end-processed intron-containing tRNAs. D) $tRNA^{Pro}_{UGG}$ 5'exon using DIG labeled probe SM18 shows probe hybridizes to both the mature tRNA

species and the unknown fast migrating RNA species in all lanes including wild-type that has not been exposed to heat shock for 2 hours with equal amounts E) $tRNA^{Pro}_{UGG}$ intron using DIG labeled probe SM19 shows probe does not hybridize to the mature tRNA species or the unknown fast migrating RNA species in all lanes including wild-type that has not been exposed to heat shock. F) $tRNA^{Pro}_{UGG}$ 3'exon using DIG labeded probe SM22 shows probe hybridizes to both the mature tRNA species and the unknown fast migrating RNA species in all lanes including wild-type that has not been exposed to heat shock for 2 hours. However, the probe hybridizes more with the mature tRNA species compares to the unknown RNA species.

Figure 10 Mutations in GlnRS, PheRS and ThrRS cause accumulation of end-processed intron-containing $tRNA^{Lys}_{UUU}$. Northern analysis of $tRNA^{Lys}_{UUU}$ employing DIG probe SM15. Slower migrating initial precursor transcript tRNA (Pa), Faster migrating initial precursor transcript tRNA (Pb), end-processed intron-containing tRNA (I), Mature tRNA (M). I/P ratio of species I divided by (Pa+Pb) and normalized to wild-type cells grown under same conditions. Lane numbers marked in red are mutant aaRS which consistently cause accumulation of end-processed intron-containing tRNAs.

Figure 11 Mutation in PheRS causes accumulation of end-processed intron-containing $tRNA^{Ser}_{CGA}$. Northern analysis of $tRNA^{Ser}_{CGA}$ employing DIG probe SM20. Initial precursor transcript tRNA (P), end-processed intron-containing tRNA (I), Mature tRNA (M). I/P ratio of species I divided by P and normalized to wild-type cells grown under same conditions. Lane numbers marked in red are

mutant aaRS which consistently cause accumulation of end-processed intron-containing tRNAs.

Figure 12 No mutant aaRS causes an accumulation of end-processed intron-containing $tRNA^{Ser}_{GCU}$. Northern analysis of $tRNA^{Ser}_{GCU}$ employing DIG probe SM21. Initial precursor transcript tRNA (P), end-processed intron-containing tRNA (I), Mature tRNA (M). I/P ratio of species I divided by P and normalized to wild-type cells grown under same conditions. Lane numbers marked in red are mutant aaRS which consistently cause accumulation of end-processed intron-containing tRNAs.

Figure 13 $tRNA^{Ile}_{UAU}$ does not accumulate in the nucleus if *frs2* cells. Wild-type, *rna1-1, sen2-42,* and *frs2* cells were cultured at 23°C, shifted to 37°C, and harvested at 0 and 2 hr after the temperature shift A) The localization of all $tRNA^{Ile}_{UAU}$ species was examined by FISH using probe SRIM03. Nuclei were visualized by DAPI staining. Arrowheads highlight unknown clumps of tRNA accumulation in cytoplasm B) The localization of intron-containing $tRNA^{Ile}_{UAU}$ species was examined by FISH using probe SRIM04. Nuclei were visualized by DAPI staining. Arrowheads highlight unknown clumps of tRNA accumulation in cytoplasm

Figure 14 Mutant aaRS do not cause a charging defect for non-cognate tRNAs. Acid-Urea gel conducted by Dr. Kunal Chatterjee (Unpublished). Wild-type and *ths1-1* cells were cultured at 23°C and either allowed to remain at 23°C for an additional two hours or shifted to 37°C for 0 or 2 hr before harvesting. All tRNAs in both the wild-type and mutant cells at the permissive pH of 4.4 show a stable

bond between the $tRNA^{Ile}_{UAU}$ and amino acid as compared to the unstable bonds seen in all tRNAs at the non-permissive pH of 7.5.

Table 1 Oligonucleotides For Northern Analysis		
Name	tRNA homology	Sequence
JW0048	$tRNA^{Ile}_{UAU}$- Full 5'-exon, 30nt intron	5'-GGCACAGAAACTTCGGAAACCGAATGTTGCTATAAGCACGAAGCTCTAACCACTGAGCTACACGAGC -3'
KC0031	$tRNA^{Tyr}_{GUA}$ – 22nt 5'-exon, full intron, 14nt 3'exon	5'-CCCGATCTCAAGATTTCGTAGTGATAAATTACAGTCTTGCGCCTTAAACC-3'
SM10	$tRNA^{Phe}_{GAA}$ -21nt 5'exon, full intron	5'-TAACTTGACCGAAGTATTTCTTCAGTCTGGCGCTCTCCC-3'
SM11	$tRNA^{Trp}_{CCA}$ -20nt 5'exon, 21nt intron	5'-CGTGGAATTTCCAAGATTTAATTGGAGTCGAAAGCTCTACC-3'
SM12	$tRNA^{Leu}_{CAA}$ -23nt 5'exon, 22nt intron	5'-GTTAACTGCGGTCAAGATATTTCTTGAATCAGGCGCCTTAGACCG-3'
SM13	$tRNA^{Leu}_{UAG}$ -21nt 5'exon, 20nt intron	5'-ATTTTAGAGGTTAAATCCACCTAAATCTGACGCCTTAAACC-3'
SM14	$tRNA^{Pro}_{UGG}$ -20nt 5'exon, 20nt intron	5'-CCTGTTTAGGCAGGAAGTCGCCCAAAGCGAGAATCATACC-3'
SM15	$tRNA^{Lys}_{UUU}$ – 20 nt5'exon, 22nt intron	5'-CCTTGCTTAAGCAAATGCGCTTAAAAGCCGAACGCTCTACC-3'
SM18	$tRNA^{Pro}_{UGG}$ – Full 5'exon	5'-CCCAAAGCGAGAATCATACCACTAGACCACACGCCC-3'
SM19	$tRNA^{Pro}_{UGG}$ –Full intron	5'- TGCTTTGTCTTCCTGTTTAGGCAGGAAGTCG-3'
SM20	$tRNA^{Ser}_{CGA}$ –Full 5'exon, full intron	5'-AGCCGAACTTTTTATTCCATTCGAGTCTCTCGCCTTAACCACTCGGCCATAGTGCC-3'
SM21	$tRNA^{Ser}_{GCU}$ – Full 5'exon, full intron	5'-AATTGCTTTTCTGAGGAAATAGCAGGGCATCGCCTTAACCACTCGGCCACTGGGAC-3'
SM22	$tRNA^{Pro}_{UGG}$ –Full 3'exon	5'-GGGGGGCGAGCTGGGAATTGAACCCAGGGCCTCTCGCA-3'
Oligonucleotides for Fluorescent *in situ* hybridization		
SRIM04	$tRNA^{Ile}_{UAU}$ mature sequence	5'-GTGGGGATTGAACCCACGACGGTCGCGTTATAAGCACGAAGCTCTAACCACTGAGCT ACA-3'
SRIM03	$tRNA^{Ile}_{UAU}$ intron sequence	5'-CGTTGCTTTTAAAGGCCTGTTTGAAAGGTCTTTGGCACAGAAACTTCGGAAACCGAAT GTTGCTAT-3'

Oligonucleotides used for hybridization required by Northern analysis and FISH

Table 2 Northern Analysis organizational chart

Northern Lane #	aaRS mutant gene	aaRS	Corresponding aa	ts collection	Class
1	*ala1-1*	AlaRS	Alanine	Boone	II
2	*YDR341C*	ArgRS	Arginine	Hieter	I
3	*ded81*	AsnRS	Asparagine	Hieter	II
4	*YNL427W*	CysRS	Cysteine	Hieter	I
5	*gus1*	GluRS	Glutamate	Hieter	I
6	*gln4*	GlnRS	Glutamine	Hieter	I
7	*grs1*	GlyRS	Glycine	Hieter	II
8	*hts1-1*	HisRS	Histidine	Boone	II
9	*ils1-1*	IleRS	Isoleucine	Boone	I
10	*cdc60-ts*	LeuRS	Leucine	Boone	I
11	*mes1-1*	MetRS	Methionine	Boone	I
12	*frs2*	PheRS	Phenylalanine	Hieter	II
13	*YHR020W*	ProRS	Proline	Hieter	II
14	*ths1*	ThrRS	Threonine	Hieter	II
15	*tys1-1*	TyrRS	Tryosine	Boone	I
16	*vas1*	ValRS	Valine	Hieter	I
17	WT-BY4741	N/A	N/A	N/A	N/A
18	Positive control *sen2-42*	N/A	N/A	Boone	N/A

Table 3. Comprehensive chart of all mutant aaRS that give rise to tRNA splicing defect

		tRNAs									
aaRS ts mutants/corresponding aaRS		Ile	Tyr	Phe	Trp	Leu_{CAA}	Leu_{UAG}	Pro	Lys	Ser_{CGA}	Ser_{GCU}
	ala1/ alaRS										
	YDR341C/ argRS		+			+					
	ded81/ asnRS		+								
	YNL247W/ cysRS		+								
	gus1/ gluRS										
	gln4/ glnRS	+		+	+	+	+	+	+		
	grs1/ glyRS		+	+							
	hts1/ hisRS		+			+	+				
	ils1/ ileRS	+	+		+	+	+				
	*cdc60 /*leuRS	+			+	+					
	mes1/ metRS										
	frs2/ pheRS	+	+	+	+	+	+	+	+	+	
	YHR020W/ proRS										
	ths1/ thrRS	+	+	+	+	+	+	+	+		
	tys1/ tyrRS										
	vas1/ valRS										

See Figures 3-12 for data leading to conclusions drawn

Figure 1.

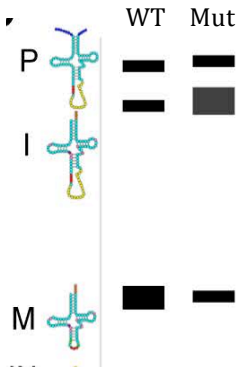


Figure 2.

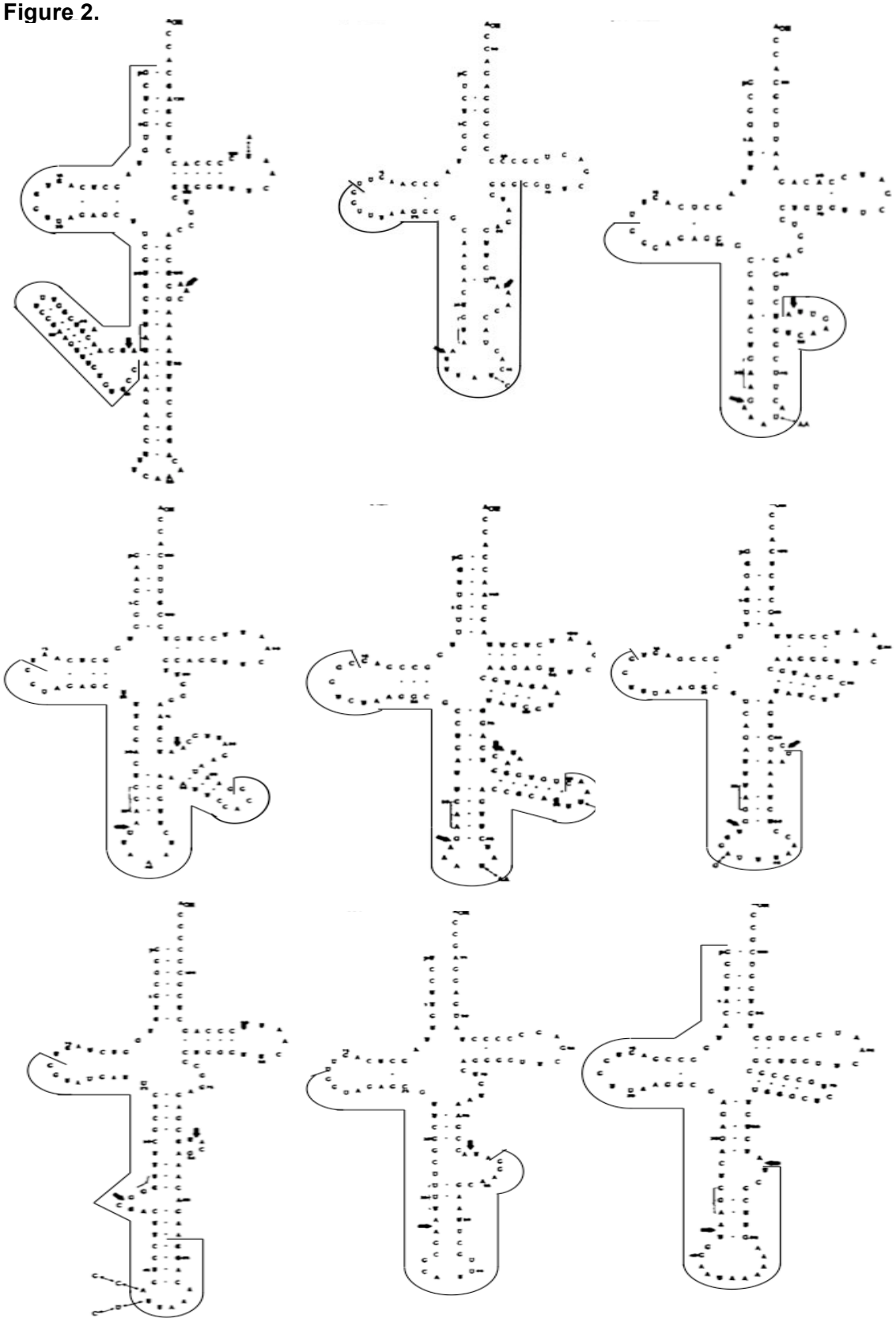


Figure 3 $tRNA^{Ile}_{UAU}$ Northern Analysis

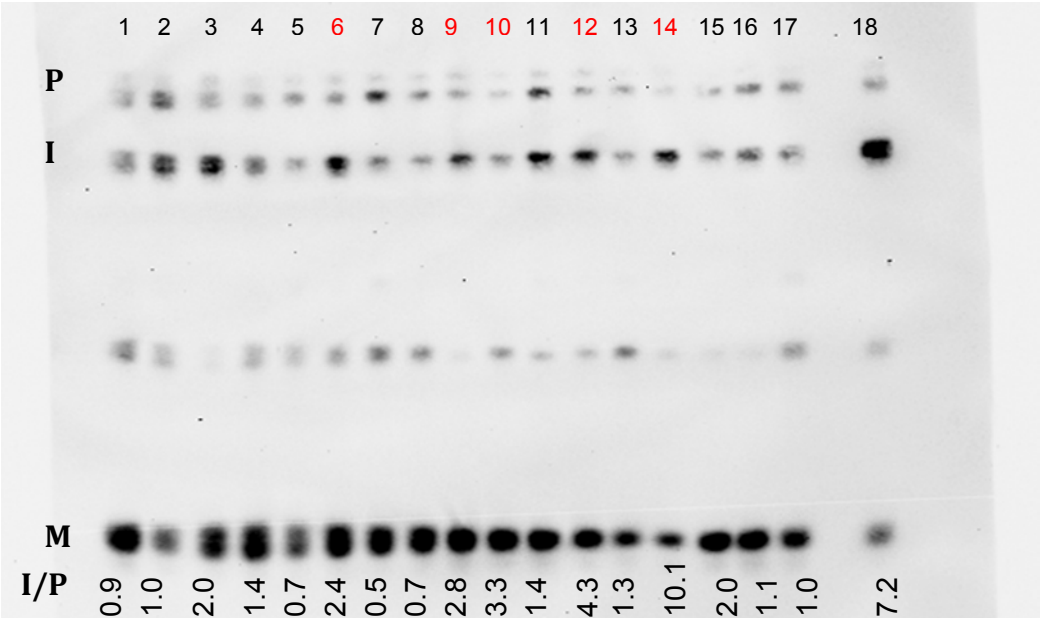


Figure 4 $tRNA^{Tyr}_{GUA}$ Northern Analysis

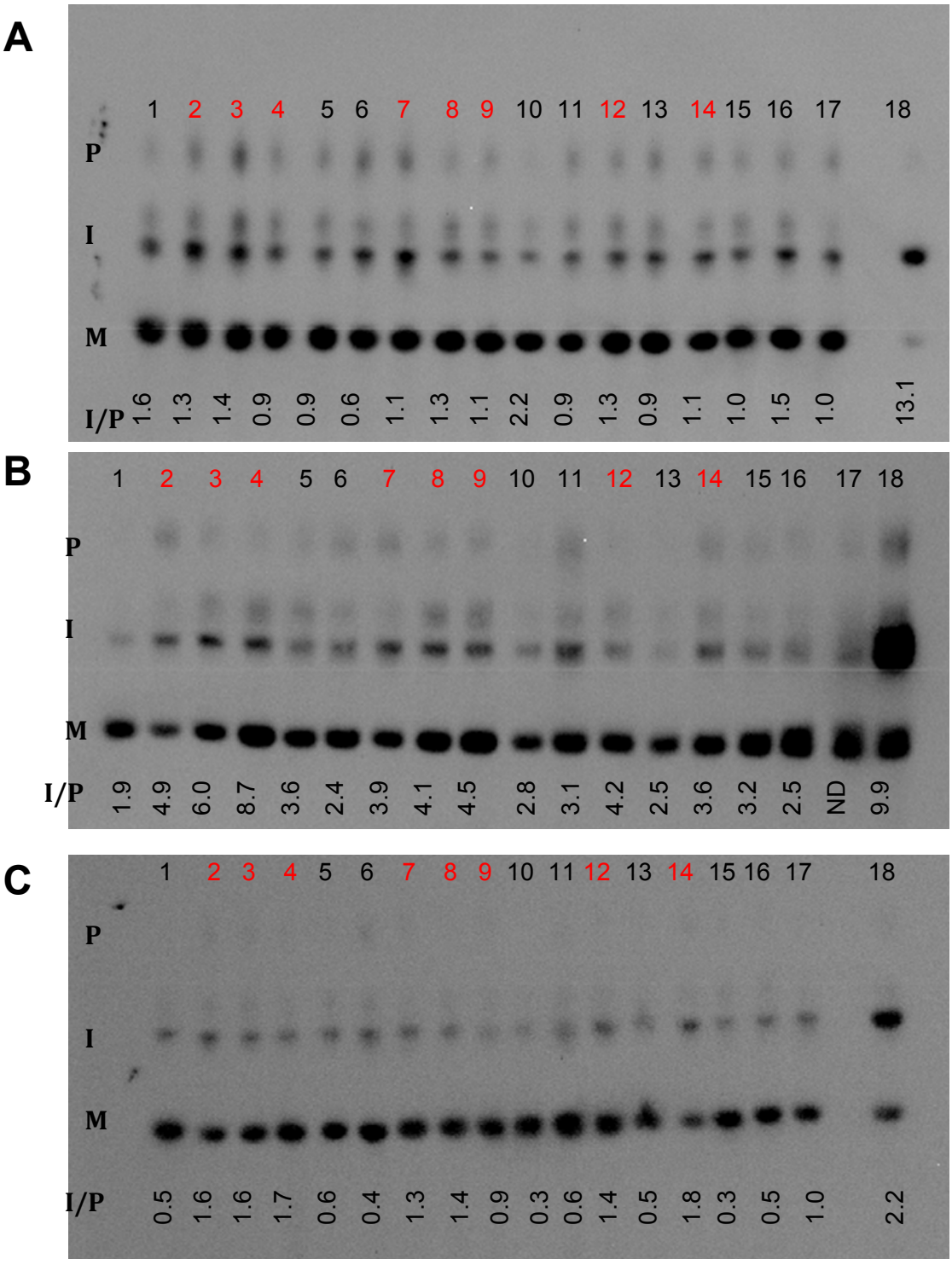


Figure 5 $tRNA^{Phe}_{GAA}$ Northern Analysis

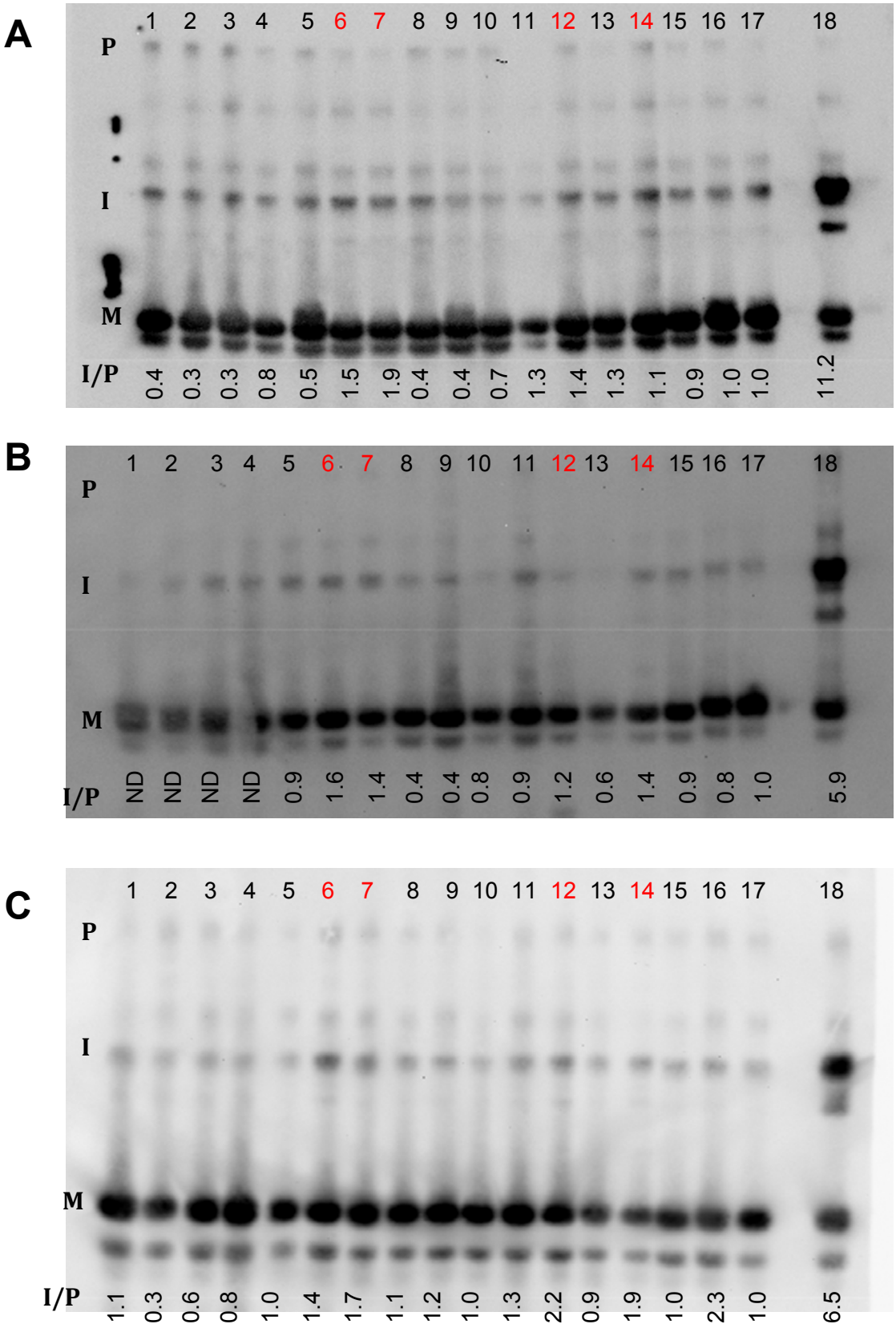


Figure 6 $tRNA^{Trp}_{CCA}$ Northern Analysis

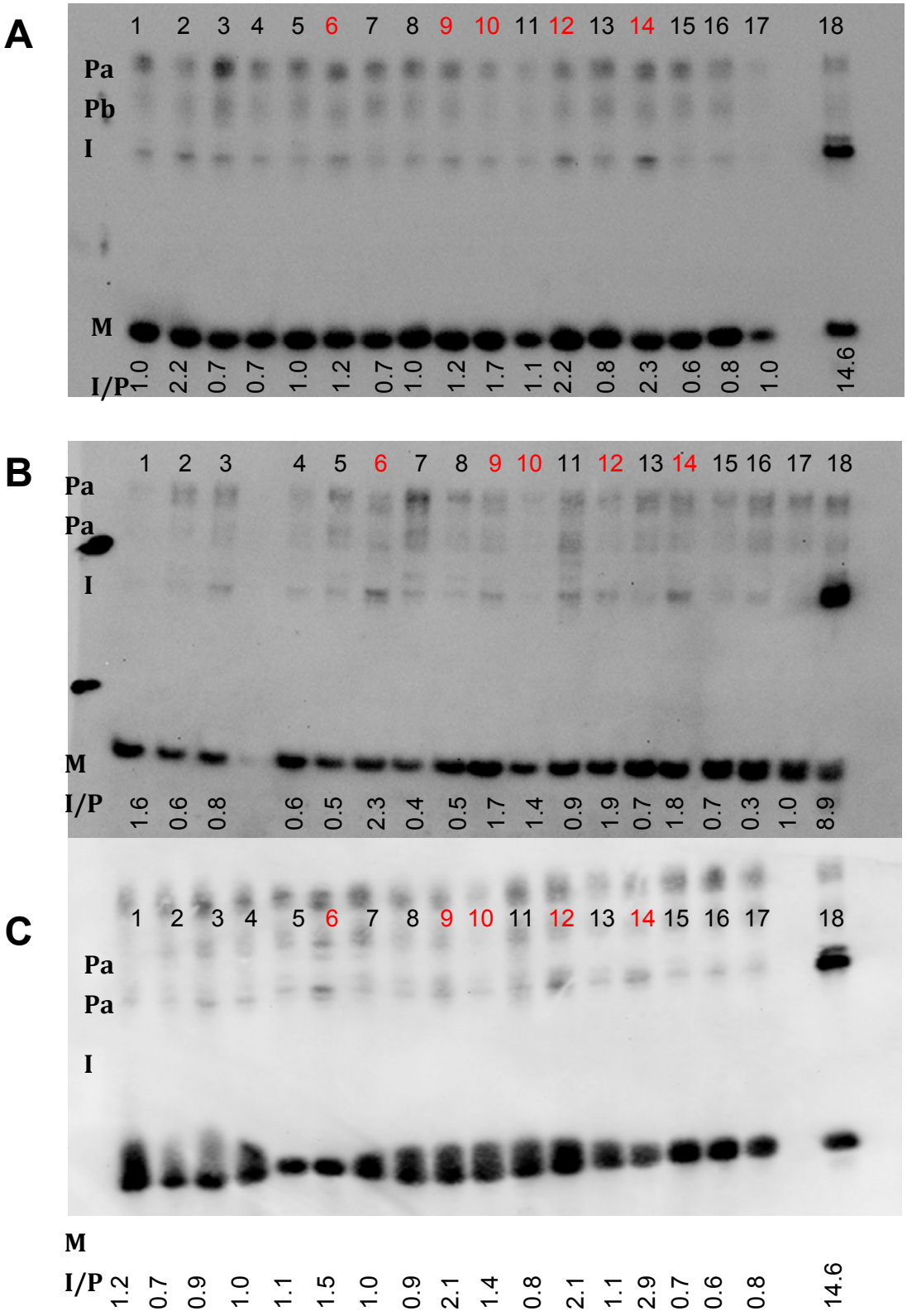


Figure 7 $tRNA^{Leu}_{CAA}$ Northern Analysis

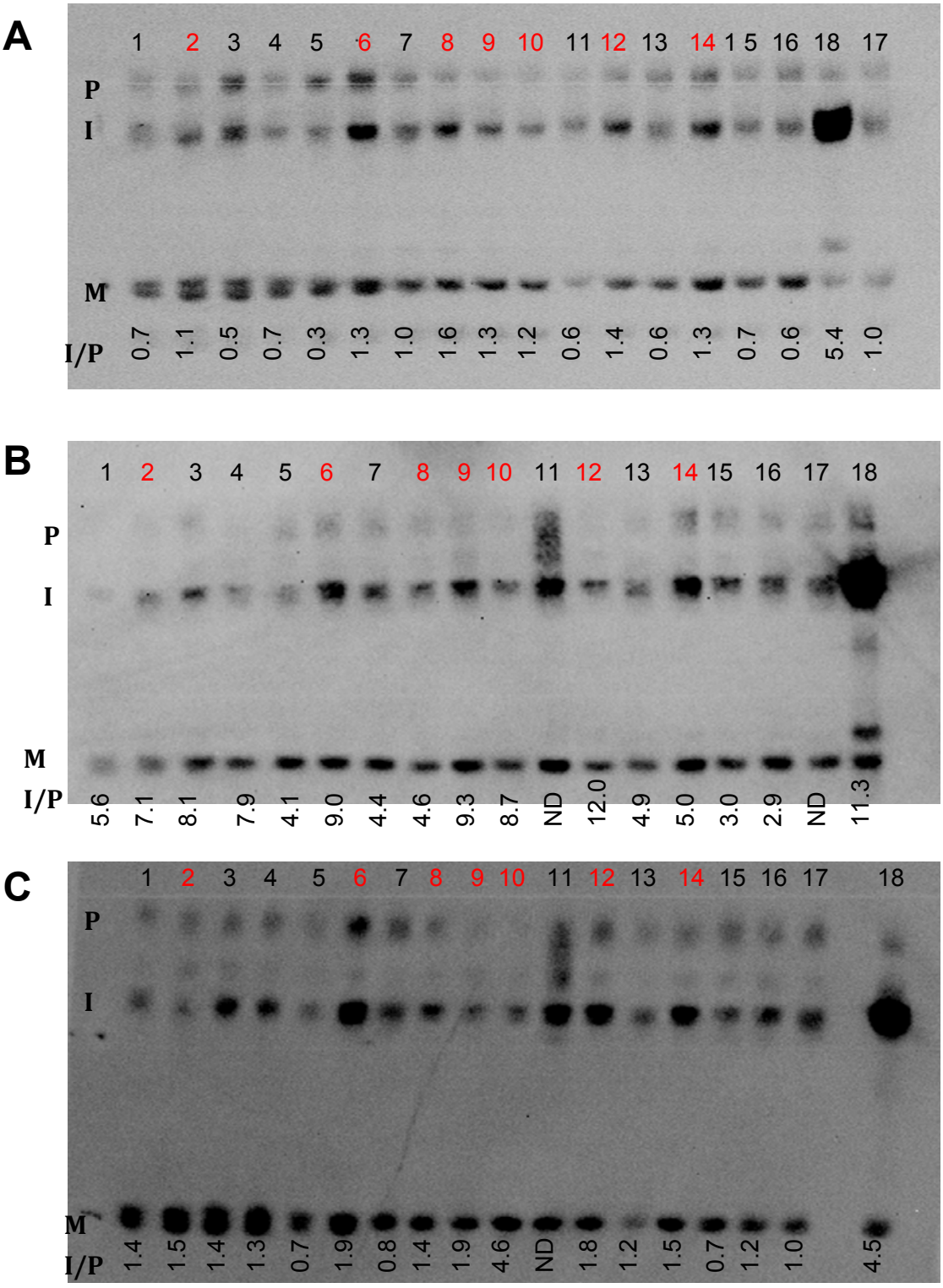


Figure 8 $tRNA^{Leu}_{UAG}$ Northern Analysis

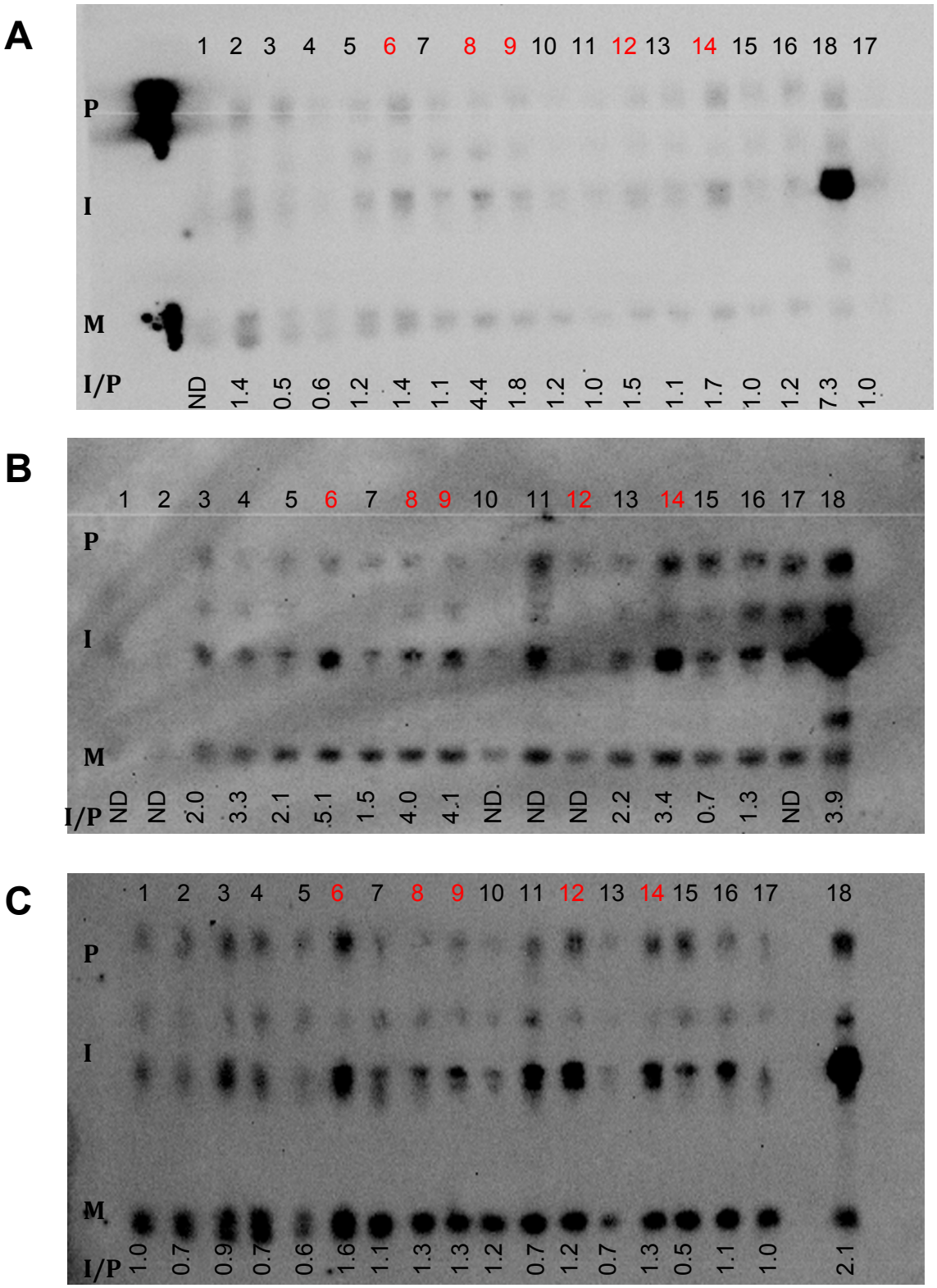
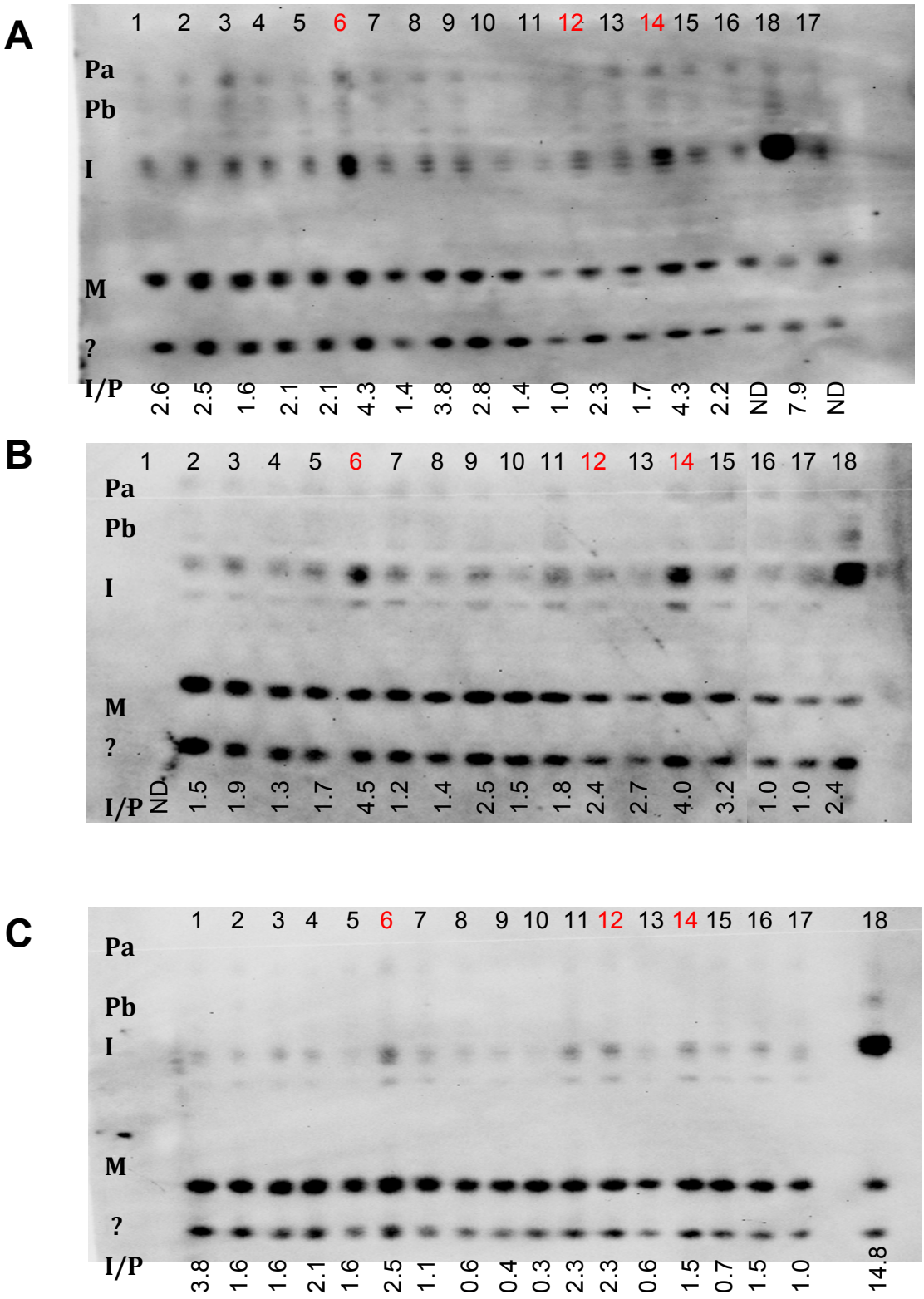
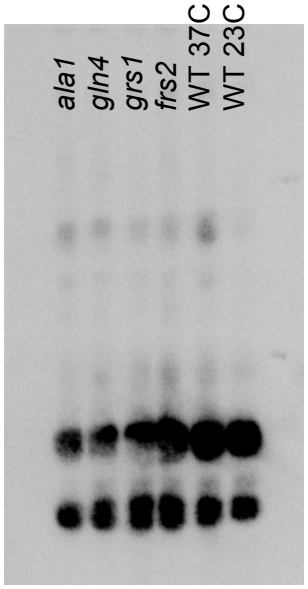


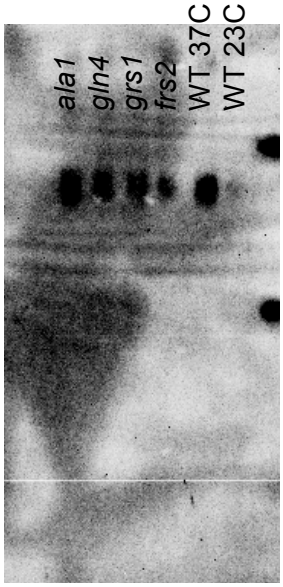
Figure 9 $tRNA^{Pro}_{UGG}$ Northern Analysis



D 5’ exon



E intron



F 3’ exon

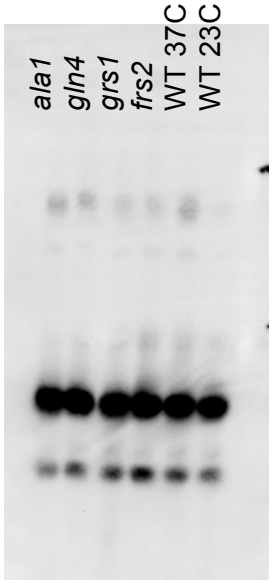


Figure 10 $tRNA^{Lys}_{UUU}$ Northern Analysis

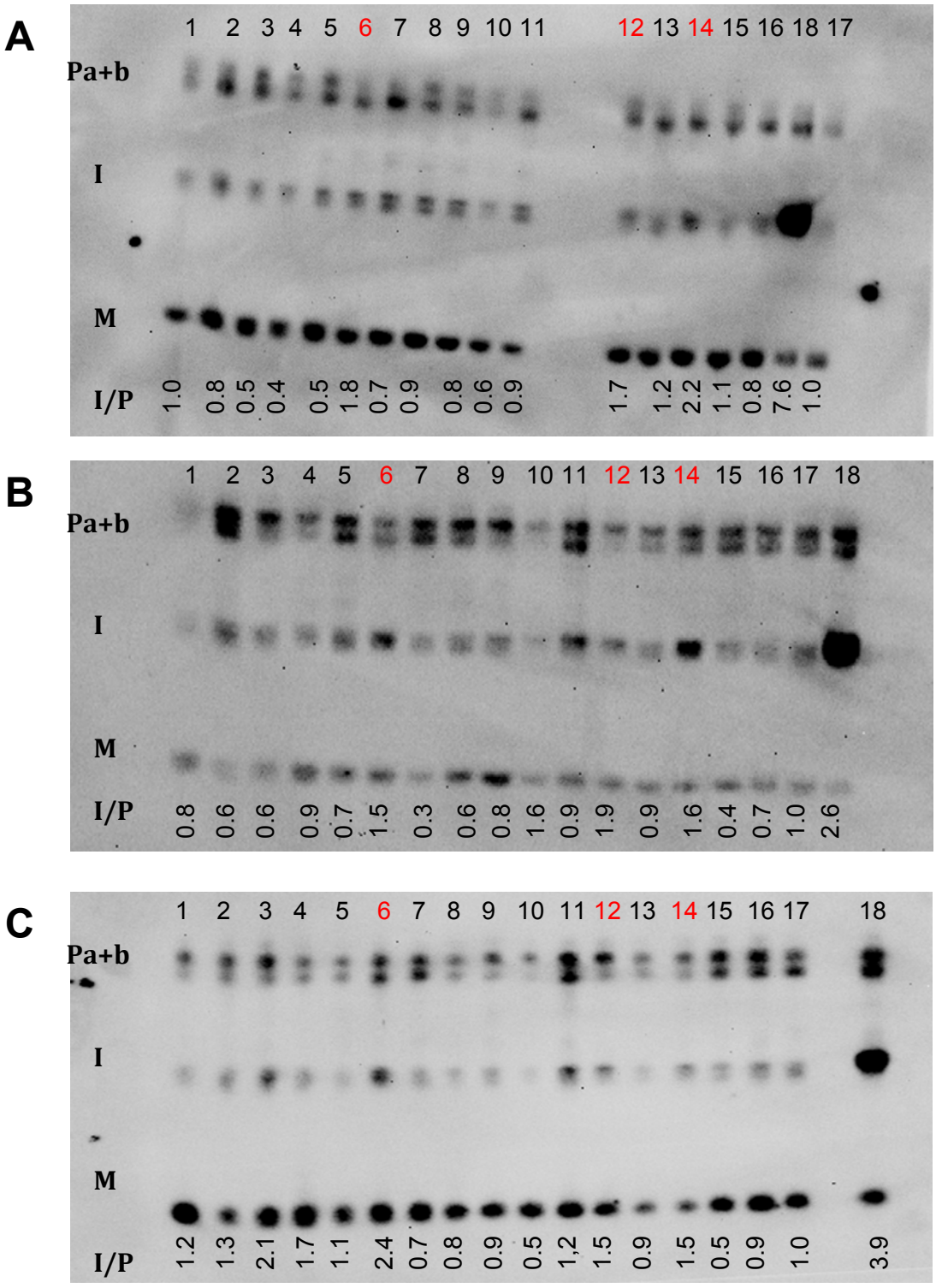


Figure 11 $tRNA^{Ser}_{CGA}$ Northern Analysis

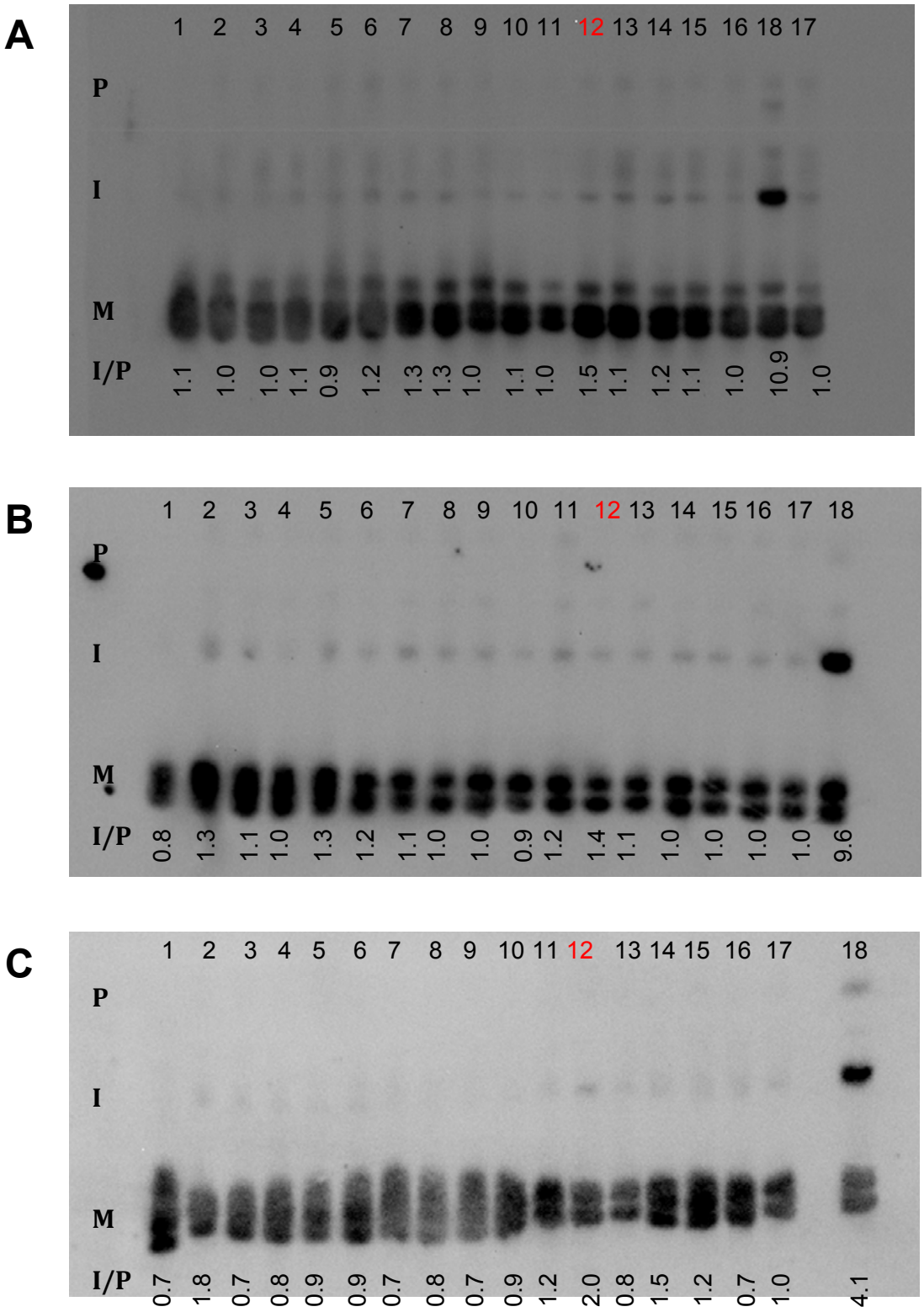


Figure 12 $tRNA^{Ser}_{GCU}$ Northern Analysis

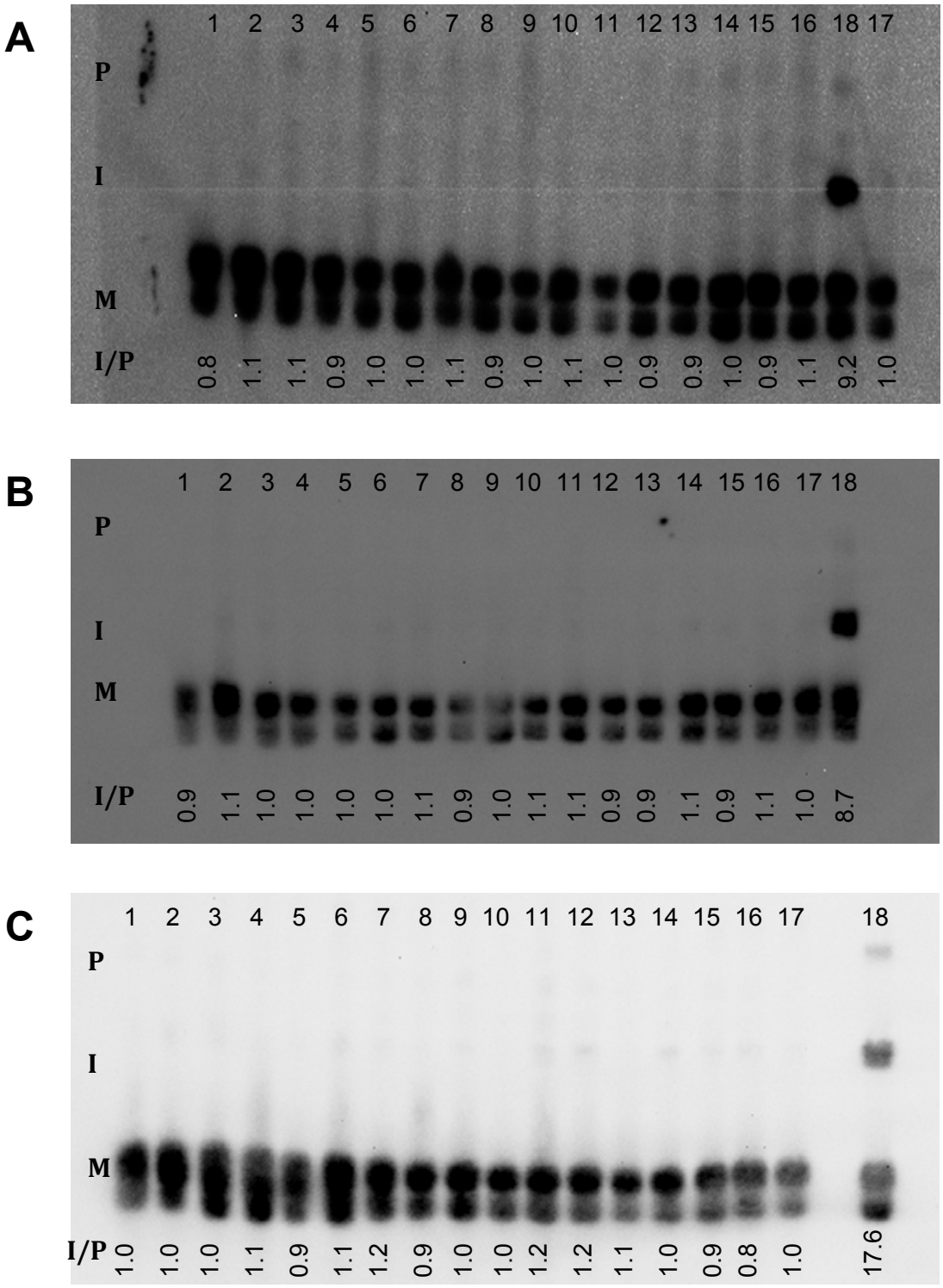


Figure 13.

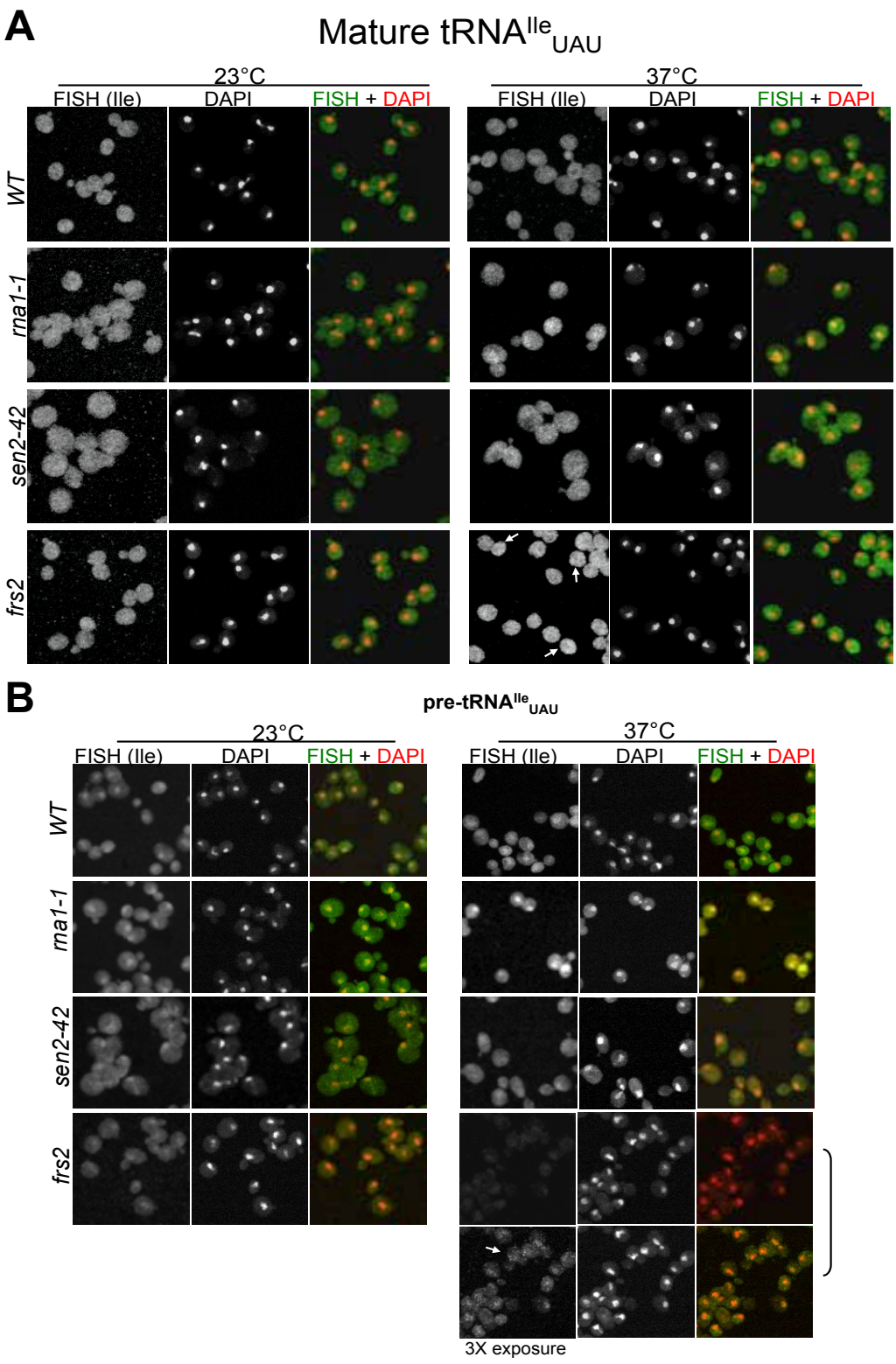
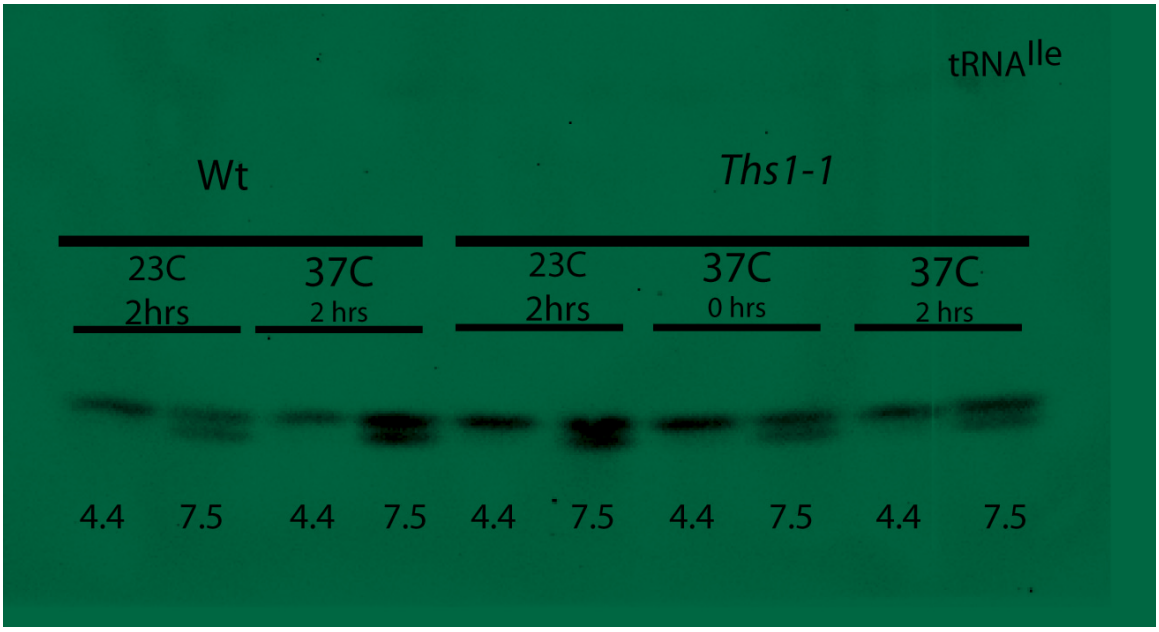

A
Mature tRNA$^{Ile}_{UAU}$
23°C
37°C
FISH (Ile)
DAPI
FISH + DAPI
WT
rna1-1
sen2-42
frs2
B
pre-tRNA$^{Ile}_{UAU}$
3X exposure

Figure 14


tRNA^{Ile}
Wt
Ths1-1
23C
2hrs
37C
2 hrs
23C
2hrs
37C
0 hrs
37C
2 hrs
4.4
7.5
4.4
7.5
4.4
7.5
4.4
7.5
4.4
7.5